

Efficacy of Oseltamivir Therapy in Ferrets Inoculated with Different Clades of H5N1 Influenza Virus[▽]

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Highly pathogenic H5N1 influenza viruses have infected an increasing number of humans in Asia, with high mortality rates and the emergence of multiple distinguishable clades. It is not known whether antiviral drugs that are effective against contemporary human influenza viruses will be effective against systemically replicating viruses, such as these pathogens. Therefore, we evaluated the use of the neuraminidase (NA) inhibitor oseltamivir for early postexposure prophylaxis and for treatment in ferrets exposed to representatives of two clades of H5N1 virus with markedly different pathogenicities in ferrets. Ferrets were protected from lethal infection with the A/Vietnam/1203/04 (H5N1) virus by oseltamivir (5 mg/kg of body weight/day) given 4 h after virus inoculation, but higher daily doses (25 mg/kg) were required for treatment when it was initiated 24 h after virus inoculation. For the treatment of ferrets inoculated with the less pathogenic A/Turkey/15/06 (H5N1) virus, 10 mg/kg/day of oseltamivir was sufficient to reduce the lethargy of the animals, significantly inhibit inflammation in the upper respiratory tract, and block virus spread to the internal organs. Importantly, all ferrets that survived the initial infection were rechallenged with homologous virus after 21 days and were completely protected from infection. Direct sequencing of the NA or HA1 gene segments in viruses isolated from ferret after treatment showed no amino acid substitutions known to cause drug resistance in conserved residues. Thus, early oseltamivir treatment is crucial for protection against highly pathogenic H5N1 viruses and the higher dose may be needed for the treatment of more virulent viruses.

Human infections with highly pathogenic avian H5N1 influenza viruses were first documented in 1997 (4, 6, 38). Two fatal human cases of H5N1 infection were reported in 2003 in Southeast Asia (33) and were followed by multiple introductions of H5N1 viruses into humans in different parts of the world in 2004 to 2006 (7, 15). At this time, human H5N1 influenza virus infection has been documented in 10 Eurasian countries, with a mortality rate of >50% (46). Although person-to-person transmission remains limited (43), the rapid evolution, genetic diversity, unprecedented geographic spread, and changing ecology of the virus raise concerns of a pandemic.

Clinical management of human H5N1 infection is uncertain. Influenza-like illness and conjunctivitis are observed early in the course of disease (1, 49), which often progresses to pneumonia and lethal acute respiratory distress syndrome or multiorgan failure (15, 43). Clinical manifestations may include gastrointestinal, pulmonary, and central nervous system symptoms (7). There is limited information about the extrapulmonary replication of H5N1 influenza viruses in humans, but virus has been isolated from cerebrospinal fluid, feces, throat specimens, and serum (7), suggesting that the virus spreads system-

ically and that multiple-organ involvement plays a role in the high mortality rate.

Although strain-specific vaccines are considered the best preventive therapy, antiviral drugs will clearly be the most important short-term resource at the start of a pandemic. The M2 ion channel blocker amantadine, one of the two available classes of influenza-specific drugs, was used effectively against pandemic H3N2 influenza in 1968 (37). However, H5N1 viruses isolated in China (27, 33), Thailand, Cambodia, and Vietnam (11, 15) have asparagine at position 31 of the M2 protein and therefore are resistant to M2 inhibitors. Recent H5N1 isolates from Indonesia, China, Mongolia, Russia, Iraq, Egypt, and Turkey are susceptible to amantadine (2). Therefore, the M2 ion channel blockers can be used only against susceptible variants.

Oseltamivir, a neuraminidase (NA) inhibitor (the other class of anti-influenza drugs), was used successfully to control the transmission of highly pathogenic avian H7N7 influenza virus in The Netherlands (23). It has also been used in patients infected with H5N1 virus in Asia but generally only 5 to 10 days after the onset of symptoms and at suboptimal doses (3, 47). There are only two case reports describing the emergence of oseltamivir-resistant H5N1 during or after therapy (8, 24). Most of the resistant clones carried an H274Y NA mutation, although some had an N294S NA mutation. In one case, a mixture of drug-resistant and drug-sensitive virus clones was isolated (24).

It is not known whether antiviral drugs that are effective against contemporary human influenza viruses will be effective

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against systemically replicating H5N1 viruses. In the absence of human trials of antiviral drugs against these viruses, animal models offer the best experimental approach. Both of the NA inhibitors (zanamivir and oseltamivir) increase survival in animal models of H5N1 infection. Zanamivir protected mice against lethal challenge with A/HK/156/97 (H5N1) influenza virus and protected chickens against highly pathogenic A/chick/Victoria/1/85 (H7N7) virus (12, 13). The orally administered NA inhibitor oseltamivir was an effective treatment for H5N1 and H9N2 influenza virus infection in mice (9, 26). Recent studies showed a significantly dose-dependent effect against A/Vietnam/1203/04 (H5N1) virus in mice and a need for a higher-dose, more prolonged treatment for the more pathogenic new antigenic variant of A/Vietnam/1203/04 virus (48).

Ferrets are naturally susceptible to influenza viruses and have been used to test the efficacy of NA inhibitors against contemporary H1N1 and H3N2 influenza viruses (31, 40). Ferrets were recently established as an appropriate animal model for studying the pathogenicity of H5N1 influenza viruses, and it was suggested that manifestations of clinical symptoms of infection in that animal model closely reflect the signs of disease observed in humans (10, 29, 50). To our knowledge, the ferret model has not been used to evaluate the efficacy of antiviral drugs against highly pathogenic H5N1 viruses.

In the present study, we determined the efficacy of oseltamivir for postexposure prophylaxis and for delayed treatment (24 h after inoculation) of H5N1 influenza virus infection in ferrets. We used two H5N1 viruses isolated from fatally infected humans in different geographical areas. The viruses represent two different clades on the H5 HA phylogenetic tree: clade 1 (A/Vietnam/1203/04 [H5N1]) and the more diverse clade 2 (A/Turkey/15/06 [H5N1]) (46). We determined the dose of oseltamivir required to protect against lethal infection and to ameliorate the duration and severity of disease with respect to virus pathogenicity, H5N1 virus load, the time of initiation of treatment, and the emergence of oseltamivir-resistant variants.

MATERIALS AND METHODS

Compound. The NA inhibitor oseltamivir carboxylate, the active metabolite of oseltamivir, and oseltamivir phosphate ([3R,4R,5S]-4-acetamido-5-amino-3-[1-ethylpropoxy]-1-cyclohexane-1-carboxylic acid) were provided by F. Hoffmann-La Roche Ltd. (Basel, Switzerland) as lyophilized powder maintained at 4°C. The compounds were dissolved in sterile distilled water at a concentration of 1 mg/ml, and aliquots were kept frozen at -70°C until used.

Viruses and cells. The H5N1 influenza viruses A/Vietnam/1203/04 and A/Turkey/15/06 were obtained from the World Health Organization collaborating laboratories. Stock viruses were grown in the allantoic cavities of 9-day-old embryonated chicken eggs for 32 h at 36°C, and aliquots were stored at -70°C until used. Virus titer was determined by calculating the 50% egg infectious dose (EID₅₀) per ml of virus stock (34). Experiments with highly pathogenic H5N1 influenza viruses were conducted in an animal biosafety level 3+ containment facility approved by the U.S. Department of Agriculture and the U.S. Centers for Disease Control and Prevention.

Madin-Darby canine kidney (MDCK) cells were obtained from the American Type Culture Collection (Manassas, VA) and were grown in minimal essential medium supplemented with 5% fetal bovine serum, 5 mM L-glutamine, 0.2% sodium bicarbonate, 100 U/ml penicillin, 100 µg/ml streptomycin sulfate, and 100 µg/ml kanamycin sulfate in a humidified atmosphere of 5% CO₂.

Plaque assay. Plaque assays were performed as described previously (14) with MDCK cells to determine the virus yield and the diameter of plaques produced by H5N1 viruses.

Drug susceptibility in tissue culture assay. The drug susceptibility of H5N1 viruses was determined by plaque reduction assay (14). MDCK cells were inoculated with virus diluted in minimal essential medium to yield 80 to 100 plaques per well and then were overlaid with infection medium containing oseltamivir carboxylate (0.001 to 100 µM). The results were recorded after 3 days of incubation at 37°C. At least three to four independent experiments were performed to determine the concentration of compound required to reduce the plaque size by 50%, relative to the plaque size in untreated wells (EC₅₀). Drug toxicity was determined by visually comparing cytopathic changes in infected cells with those in uninfected cells in duplicate wells.

Animals. Young adult male ferrets 4 to 5 months of age were obtained from Marshall's Farms (North Rose, NY). The ferrets were seronegative for influenza A H1N1 and H5N1 and for influenza B viruses but possessed hemagglutinin (HA) inhibition (HI) antibodies (1:80 to 1:160) against currently circulating A/New York/55/04 (H3N2) virus. All animal experiments were conducted under applicable laws and guidelines and after approval by the Animal Care and Use Committee of St. Jude Children's Research Hospital.

Assessment of drug efficacy in ferrets. Ferrets were lightly anesthetized with isoflurane and inoculated intranasally with infectious virus in 1.0 ml phosphate-buffered saline (PBS). In tests of postexposure prophylaxis, groups of five ferrets were inoculated with A/Vietnam/1203/04 (H5N1) virus at a dose of either 10 or 10² EID₅₀. Three animals were observed for survival and clinical signs of infection, and two were sacrificed to determine virus titers in the internal organs. Oseltamivir phosphate (oseltamivir) was mixed 1:1 with sterile sugar syrup and administered orally by syringe toward the rear of the tongue, which allowed the ferrets to swallow the drug without discomfort. Oseltamivir treatment (5 mg/kg of body weight/day given as two daily doses of 2.5 mg/kg for 5 days) began 4 h after virus inoculation. Control inoculated ferrets received sterile PBS mixed 1:1 with sterile sugar syrup (placebo) on the same schedule. Clinical signs of infection, relative inactivity index (35), weight, and temperature were recorded daily. Assessment of the activity level was done based on the following scoring system: 0, alert and playful; 1, alert but playful only when stimulated; 2, alert but not playful when stimulated; and 3, neither alert nor playful when stimulated. Animals that showed signs of severe disease and >25% weight loss were euthanized. Body temperature was measured by subcutaneous implantable temperature transponders (Bio Medic Data Systems Inc., Seaford, DE).

The efficacy of delayed treatment with oseltamivir was studied in groups of five ferrets inoculated with either 10² EID₅₀ of A/Vietnam/1203/04 or 10⁶ EID₅₀ of A/Turkey/15/06 virus. Three animals were observed for survival and clinical signs of infection, and two were sacrificed to determine virus titers in the internal organs. Oseltamivir treatment (10 or 25 mg/kg/day in twice-daily doses for 5 days) was initiated 24 h after virus inoculation, and the animals were observed as described above. In control, uninfected ferrets (two animals per group) that received oseltamivir on the same schedule, no weight changes or behavior abnormalities were observed. Inflammatory cell counts were determined in the nasal washes of ferrets inoculated with A/Turkey/15/06 (H5N1) virus as described previously (30). Briefly, the nasal washes were collected and centrifuged at 2,000 rpm for 10 min. The cells' pellet was resuspended in PBS, and the cells were counted in a hemacytometer under the microscope. The total number of inflammatory cells was calculated based on the initial volume of the nasal wash. The protein concentration in cell-free nasal washes was determined with a protein reagent from Bio-Rad (Hercules, CA).

Reinfection with H5N1 virus. Three weeks after inoculation with H5N1 virus, surviving ferrets were rechallenged with homologous virus at a dose of 10² EID₅₀ of A/Vietnam/1203/04 (H5N1) and 10⁷ EID₅₀ of A/Turkey/15/06 (H5N1) virus. Clinical signs of infection, weight, and temperature were monitored daily.

Titration of virus in the upper respiratory tract. On days 3, 5, and 7 after inoculation, ferrets were anesthetized with ketamine (25 mg/kg) injected intramuscularly, and 0.5 ml sterile PBS containing antibiotics was introduced into each nostril and collected in containers. Virus was titrated in embryonated chicken eggs by injecting 0.1 ml of serial 10-fold dilutions of the sample (three eggs per dilution) and expressed as log₁₀ EID₅₀/ml.

Titration of virus in organs. Organs were collected after oseltamivir treatment was completed (on day 6 after inoculation). Two animals in each treatment and control group were euthanatized by intracardiac injection of Euthanasia V solution, and tissue samples (~0.5 g each) were collected from lung, brain, spleen, liver, and small intestine. Samples were homogenized in 1 ml sterile PBS with antibiotics, and the virus titer (log₁₀ EID₅₀/g) in embryonated chicken eggs was determined.

Serologic tests. Serum samples were collected from ferrets 3 weeks after inoculation, treated with receptor-destroying enzyme, heat inactivated at 56°C for 30 min, and tested by HI assay with 0.5% packed chicken red blood cells.

TABLE 1. Pathogenicity of the two H5N1 influenza viruses to ferrets

Virus and virus dose (EID ₅₀ /ferret) ^a	No. of ferrets that survived/ total no.	Mean day to death ± SD ^b	Relative inactivity index ^c	Avg wt loss ± SD (%) on indicated day p.i. ^d				Mean nasal wash titer ± SD (log ₁₀ EID ₅₀ /ml) on indicated day p.i. ^e		
				3	5	7	10	3	5	7
A/Vietnam/1203/04										
10	1/3	10.0 ± 0.0	0.68	5.4 ± 0.9*	11.9 ± 1.4	18.4 ± 1.4	21.4 ^f	2.9 ± 0.5	5.1 ± 0.3	4.4 ± 0.1
10 ²	0/3	7.7 ± 0.7	1.25	10.1 ± 0.7*	13.7 ± 1.4	25.2 ^f	N/A	3.8 ± 1.0	5.7 ± 0.1	6.5 ^g
10 ³	0/3	5.7 ± 0.7	1.27	15.6 ± 1.7*	23.9 ± 2.5*	27.2 ^f	N/A	4.7 ± 0.1	5.3 ± 0.3	6.5 ^g
A/Turkey/15/06										
10 ⁵	3/3	>21.0*	0.07	8.0 ± 2.3	9.2 ± 2.6*	7.4 ± 1.6*	2.5 ± 0.6*	4.5 ± 0.9	3.2 ± 0.4	2.5 ^g
10 ⁶	3/3	>21.0*	0.41	12.0 ± 1.7	19.0 ± 1.4	15.4 ± 1.6	5.0 ± 0.3	5.4 ± 0.6	4.2 ± 0.7	3.3 ^g
10 ⁷	2/3	6.0 ± 0.0	0.62	11.5 ± 1.3	22.9 ± 1.6	16.3 ± 1.5	5.3 ± 0.6	5.4 ± 0.4	4.0 ± 1.1	2.8 ± 0.4

^a Groups of three ferrets were inoculated intranasally under light anesthesia with H5N1 influenza virus in 1.0 ml of PBS.

^b The mean day to death of ferrets was determined by the Kaplan-Meier method. *, $P < 0.05$, one-way ANOVA.

^c Determined after 14 days of observation (35). Ferrets inoculated with 10² and 10³ EID₅₀/ml of A/Vietnam/1203/04 (H5N1) virus were observed for only 7 days because of mortality. Assessment of the activity level was done based on the following scoring system: 0, alert and playful; 1, alert but playful only when stimulated; 2, alert but not playful when stimulated; 3, neither alert nor playful when stimulated. The relative inactivity index before inoculation was 0.

^d The loss of weight was calculated for each ferret individually as a percentage of its weight on day 0 before virus inoculation. The values are averages for two to three animals per group. *, $P < 0.05$, one-way ANOVA.

^e The lower limit of virus detection was 0.75 log₁₀ EID₅₀/ml.

^f Results obtained from one ferret because of mortality. N/A, not applicable (all ferrets in the group died).

^g Results obtained from one ferret because of mortality (A/Vietnam/1203/04 virus) or because only one animal shed virus (A/Turkey/15/06 virus).

Immunostaining. Brain was collected 6 days postinoculation (p.i.), fixed in 10% neutral buffered formalin, and embedded in paraffin. Five-micrometer-thick tissue sections were deparaffinized, and endogenous peroxidase was inactivated in 0.3% hydrogen peroxide in absolute methanol for 15 min. Slides were incubated with 0.2% proteinase K (Chemicon, Temecula, CA) in a moist chamber at 37°C for 10 min to retrieve antigen, and nonspecific antibody binding of antibodies was blocked with 5% normal goat serum. Sections were incubated overnight with the primary antibody (1:200 dilution; anti-NP monoclonal antibodies) in PBS containing 2.5% bovine serum albumin. Control sections were incubated with vehicle. Sections were incubated with secondary goat anti-mouse antibody labeled with horseradish peroxidase (Sigma, St. Louis, MO). Nuclei were counterstained with hematoxylin, and antigen was visualized in a 3,3'-diaminobenzidine solution with hydrogen peroxide.

Virus sequence analysis. All posttreatment samples confirmed to be virus positive by virus isolation in embryonated chicken eggs were used for sequence analysis. Viral RNA was isolated from ferret nasal washes or organs by using the RNeasy mini kit (QIAGEN, Valencia, CA). Samples were reverse transcribed and analyzed by PCR using primers specific for the HA (HA1 region) and NA gene segments, as described previously (17). For clonal analysis of the virus population, we used a TOPO TA cloning kit for sequencing (Invitrogen, Carlsbad, CA) to analyze individual plaques obtained in MDCK cells. Briefly, viral RNAs were extracted from plaque samples and one-step reverse transcriptase-PCR was performed. PCR products were purified with the QIAquick PCR purification kit (QIAGEN, Valencia, CA), ligated to the pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA), and used for the transformation of TOP10 competent cells (Invitrogen, Carlsbad, CA). Plasmid DNA was prepared by using the

QIAprep spin miniprep kit (QIAGEN, Valencia, CA). Sequencing was performed by the Hartwell Center for Bioinformatics and Biotechnology at St. Jude Children's Research Hospital. The DNA template was sequenced by using rhodamine or dichlororhodamine dye terminator cycle-sequencing ready reaction kits with AmpliTaq DNA polymerase FS (Perkin-Elmer, Applied Biosystems, Inc., Foster City, CA) and synthetic oligonucleotides. Samples were analyzed in a Perkin-Elmer Applied Biosystems DNA sequencer (model 373 or 377). DNA sequences were completed and edited by using the Lasergene sequence analysis software package (DNASTAR, Madison, WI).

Statistical analysis. Virus titers in ferret organs and nasal wash samples or differences in fevers and weights were compared by analysis of variance (ANOVA) or unpaired two-tailed t test. The Kaplan-Meier method was used to estimate the probability of survival, and the log rank test was used to compare outcomes of the placebo and treatment groups (44). The proportional-hazard model was used to determine the death hazard ratio of the treatment and placebo groups (5). A probability value of 0.05 was prospectively chosen to indicate that the findings of these analyses were not the result of chance alone.

RESULTS

Pathogenicity of H5N1 influenza A/Vietnam/1203/04 and A/Turkey/15/06 viruses in ferrets. In previous studies of the pathogenicity of H5N1 influenza viruses, ferrets were inoculated with 10⁶ or 10⁷ EID₅₀ of virus; these high doses caused

TABLE 2. Effect of postexposure oseltamivir treatment on the survival of ferrets lethally challenged with A/Vietnam/1203/04 (H5N1) virus

Virus dose (EID ₅₀ /ferret)	Oseltamivir dose (mg/kg/day) ^a	No. of ferrets that survived/ total no.	Mean day to death ± SD ^b	No. of ferrets showing indicated clinical sign(s) of disease/total no.				Relative inactivity index ^f
				Wt loss ^c	Increase in temp ^c	Respiratory ^d	Neurological ^e	
10	5	3/3	>21.0*	2/3 (8.8 ± 1.9)*	1/3 (5.0) ^g *	0/3	1/3	0.17
	0	1/3	10.0 ± 0.0	3/3 (21.4 ± 1.6)	2/3 (8.6 ± 1.2)	0/3	1/3	0.68
10 ²	5	3/3	21.0*	3/3 (20.4 ± 1.9)*	1/3 (3.4) ^g *	0/3	0/3	0.34
	0	0/3	7.7 ± 0.7	3/3 (25.2 ± 1.4)	3/3 (7.6 ± 1.4)	1/3	2/3	1.25

^a Starting 4 h after inoculation, 5 mg/kg/day of oseltamivir or sterile PBS (control animals) was given orally twice daily for 5 days.

^b The mean day to death of ferrets was determined by the Kaplan-Meier method. *, $P < 0.05$, unpaired t test.

^c The maximal change in the mean value (percentage) ± SD is shown in parentheses. *, $P < 0.05$, unpaired t test.

^d Respiratory signs were sneezing, wheezing, and nasal discharge.

^e Neurological signs were hind limb paresis, ataxia, torticollis, and tremors.

^f Determined after 14 days of observation (35). Because of mortality, control ferrets were observed for only 10 days (10 EID₅₀) or 9 days (10² EID₅₀).

^g Results obtained from one ferret.

severe infection (10, 29, 50) and are not optimal for testing antiviral drugs. To optimize the experimental protocol, we first characterized the A/Vietnam/1203/04 and A/Turkey/15/06 viruses with respect to clinical signs of infection and the ability to replicate in the upper respiratory tracts of ferrets (Table 1). Inoculation with A/Vietnam/1203/04 (H5N1) virus at a dose as low as 10 EID₅₀ resulted in severe disease and the deaths of two of three animals inoculated. The ferrets became febrile, lost weight steadily, and were lethargic. Inoculation with either 10² or 10³ EID₅₀ of virus caused severe clinical signs and was lethal to all animals.

Inoculation with A/Turkey/15/06 (H5N1) influenza virus caused markedly different results. After receiving 10⁵ EID₅₀ of virus, ferrets showed modest weight loss and mild illness. All animals recovered fully and survived at day 21 (Table 1). Ferrets inoculated with 10⁶ EID₅₀ of virus showed more pronounced clinical signs of disease, and one of three ferrets inoculated with 10⁷ EID₅₀ died. Virus titers in the upper respiratory tracts on days 3, 5, and 7 p.i. were comparable across the virus doses used and tended to decrease at later stages of disease but remained detectable on day 7 p.i.

Efficacy of postexposure prophylaxis. We used two different doses of A/Vietnam/1203/04 (H5N1) virus to determine the correlation between virus input and drug efficacy. Challenge with 10 EID₅₀ caused death in two of three control ferrets on day 10 p.i., and challenge with 10² EID₅₀ caused the deaths of all inoculated control animals between days 7 and 10 p.i. All ferrets that received 5 mg/kg/day of oseltamivir 4 h after inoculation survived virus challenge, although disease was not prevented. Animals inoculated with 10 EID₅₀ of virus and treated with 5 mg/kg/day of oseltamivir showed a mean weight loss of 8.8% and remained active, although one animal exhibited neurological distress between days 7 and 9 p.i. (Table 2). Animals inoculated with 10² EID₅₀ of H5N1 virus and treated with 5 mg/kg/day of oseltamivir showed more pronounced clinical signs of illness and lost as much as 20% of their initial weight.

To assess the efficacy of oseltamivir in inhibiting virus replication in the upper respiratory tract, we collected nasal washes on days 3, 5, and 7 after inoculation (Fig. 1A). Control animals inoculated with 10² EID₅₀ of A/Vietnam/1203/04 (H5N1) shed virus at mean titers of 4.4 and 5.5 log₁₀EID₅₀/ml on days 3 and 5 p.i., respectively. On day 7 p.i., only one ferret survived in this group and shed virus at a titer of 6.5 log₁₀ EID₅₀/ml. Control ferrets inoculated with 10 EID₅₀ shed virus at a lower titers (Fig. 1A). Postexposure prophylaxis with oseltamivir 5 mg/kg/day significantly ($P < 0.05$) inhibited virus replication in the upper respiratory tract; no virus was detected in the nasal washes on days 3, 5, and 7 p.i.

To investigate the efficacy of postexposure prophylaxis in inhibiting the systemic spread of virus, we collected tissues from internal organs after completion of treatment (on day 6 p.i.). Virus was detected in the lungs, brains, livers, and spleens of both control ferrets challenged with both 10 EID₅₀ and 10² EID₅₀ of A/Vietnam/1203/04 (H5N1) virus (Fig. 1B and C). Oseltamivir treatment completely inhibited virus replication in the lungs (although samples from only one lobe were analyzed in this experiment) and small intestine. However, virus was detected in the brain, liver, and spleen of one of two animals inoculated with 10² EID₅₀. At the lower infectious dose, virus titers were significantly lower in all organs ($P <$

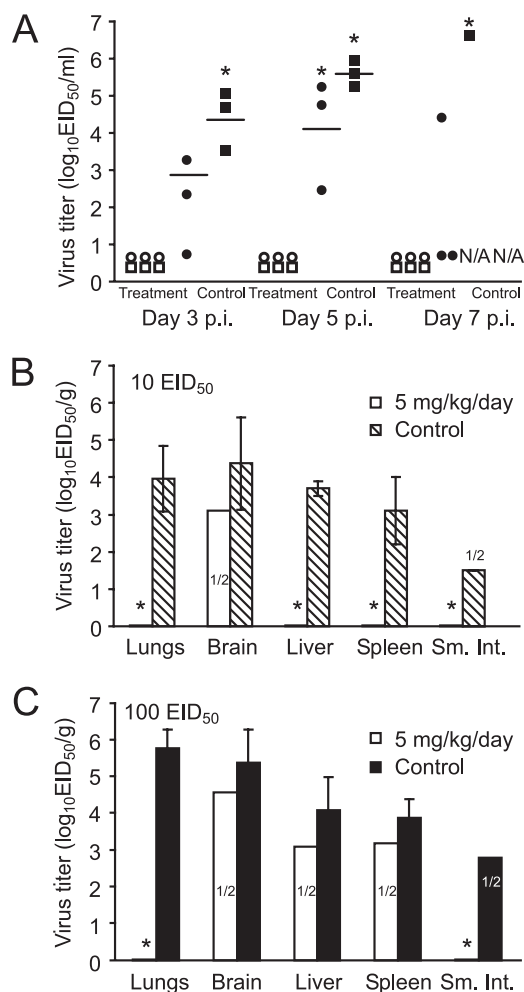


FIG. 1. Effect of postexposure oseltamivir treatment on virus titers in the nasal washes (A) and internal organs (B, C) of ferrets inoculated with A/Vietnam/1203/04 (H5N1) influenza virus. (A) Ferrets were treated with 5 mg/kg/day of oseltamivir for 5 days, beginning 4 h after inoculation with 10 EID₅₀ (○) or 10² EID₅₀ (□) of A/Vietnam/1203/04 (H5N1) virus. Control animals inoculated with 10 EID₅₀ (●) or 10² EID₅₀ (■) of virus received sterile PBS on the same schedule. Nasal washes were collected on days 3, 5, and 7 p.i. Each point represents the results from a single ferret. Lines represent mean values of positive data (limit of virus detection, 0.75 log₁₀ EID₅₀/ml). N/A, not applicable (all ferrets in the group were dead). (B, C) Virus was titrated in the lungs, brains, livers, spleens, and small intestines (Sm. Int.) of ferrets inoculated with 10 EID₅₀ (B) or 10² EID₅₀ (C) at day 6 p.i. Values are the means \pm standard deviations (SD) for two ferrets in the control group (shaded columns). In the treatment group (unshaded columns), virus was detected in one of two ferrets. *, $P < 0.05$, unpaired two-tailed t test.

0.05), except in one sample from the brain. Immunostaining of histological sections of brain tissue from control ferrets showed a wide distribution of virus-positive cells (Fig. 2A). Ferrets that received oseltamivir prophylaxis had a narrower distribution of virus in the brain and fewer virus-positive cells (Fig. 2B) than those that did not receive the treatment. Ferrets inoculated with A/Turkey/15/06 (H5N1) influenza virus had a lower level of spread to the brain (Fig. 2C and D), and these results will be discussed separately below.

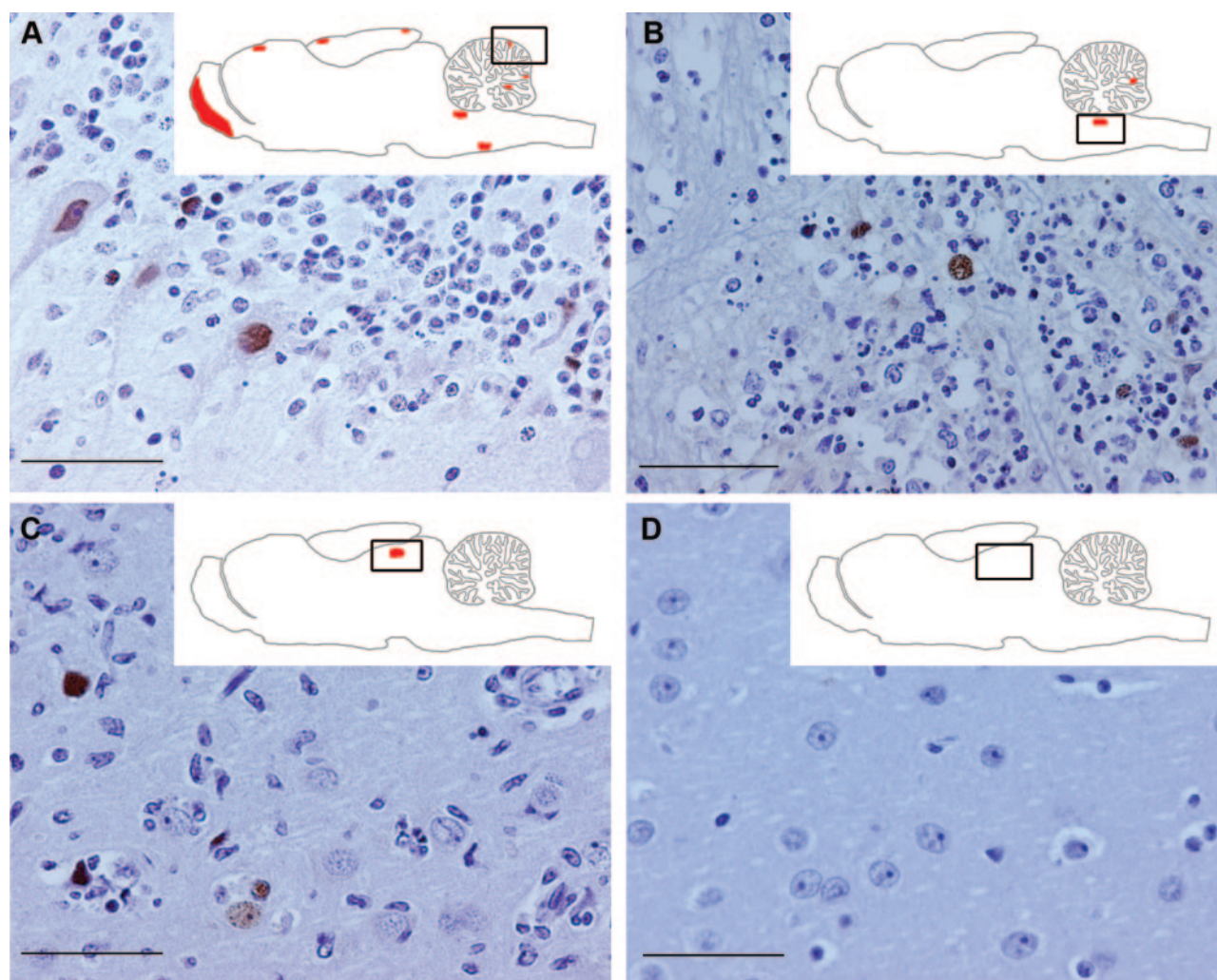


FIG. 2. Distribution of virus in the brains of control and treated ferrets on day 6 after inoculation with 10^2 EID₅₀ of A/Vietnam/1203/04 (H5N1) (A, B) or 10^6 EID₅₀ of A/Turkey/15/06 (H5N1) (C, D) influenza virus. Cells positive for viral antigen have a dark-brown granular appearance; viral distribution is shown by red shading (insets). (A) Cells positive for viral antigen are seen in the olfactory bulb in control ferrets. The inset shows the wide distribution of virus-positive cells in the brain. (B) Influenza virus antigen in an area of inflammation in the brain stem of a ferret that received oseltamivir treatment 4 h after inoculation. The inset shows a decreased spread of virus to the brain after treatment. (C) A/Turkey/15/06 (H5N1) virus was not widely distributed in the brains of control ferrets (inset). Viral antigen was detected in neurons and was associated with inflammation. (D) No virus-positive cells were detected in the brains of ferrets that received delayed (24 h p.i.) oseltamivir treatment. A/Turkey/15/06 (H5N1) virus did not spread to the brain in the presence of oseltamivir (inset). Scale bar, 50 μ m.

Efficacy of delayed treatment in ferrets inoculated with A/Vietnam/1203/04 (H5N1) virus. We next evaluated the activity of oseltamivir against established H5N1 influenza infection. Ferrets were inoculated with a lethal dose of A/Vietnam/1203/04 (H5N1) virus (10^2 EID₅₀) and treated, starting 24 h later, with either 10 or 25 mg/kg/day of oseltamivir (Table 3). All animals treated with 10 mg/kg/day experienced fever, starting on day 2 p.i., and weight loss comparable to that in control ferrets. They were extremely lethargic, and two of three animals showed severe neurological signs (uncontrolled movements and hind limb paresis). All of these ferrets died between days 7 and 8 p.i. Treatment with 25 mg/kg/day of oseltamivir resulted in 100% survival. Although this regimen did not protect ferrets completely, it markedly decreased the severity of disease and the magnitude of weight loss and fever compared to those in control animals.

The effect of treatment on nasal virus shedding was assessed on days 3, 5, and 7 p.i. (Fig. 3A). Control ferrets had mean nasal wash titers of 4.0, 4.9, and 6.1 log₁₀ EID₅₀/ml on days 3, 5, and 7 p.i., respectively. Delayed treatment with 10 mg/kg/day of oseltamivir did not inhibit virus replication in the upper respiratory tract: nasal wash titers were comparable in treated and control ferrets (Fig. 3A). Although ferrets treated with 25 mg/kg/day of oseltamivir shed virus on days 3, 5, and 7 p.i., the mean titer on day 7 p.i. was significantly lower than that in untreated animals ($P < 0.05$).

We next investigated whether delayed treatment affected H5N1 virus spread to the internal organs. In two of two control animals, virus was consistently detected in the lungs (four lobes were assayed separately), brain (two brain samples were assayed separately), liver, and spleen (Fig. 3B). Virus was detected in multiple organs when delayed treatment with 10

TABLE 3. Effect of delayed (24 h p.i.) oseltamivir treatment in ferrets lethally challenged with A/Vietnam/1203/04 (H5N1) virus

Oseltamivir dose (mg/kg/day) ^a	No. of ferrets that survived/total no.	Mean day to death \pm SD ^b	No. of ferrets showing indicated clinical sign(s) of disease/total no.				Relative inactivity index ^f
			Wt loss ^c	Increase in temp ^c	Respiratory ^d	Neurological ^e	
10	0/3	7.3 \pm 0.3	3/3 (22.1 \pm 2.9)	3/3 (5.7 \pm 1.4)	0/3	2/3	1.05
25	3/3	>21.0*	3/3 (13.4 \pm 1.7)*	3/3 (6.0 \pm 0.4)	0/3	2/3	0.30
0	0/3	6.7 \pm 0.4	3/3 (27.8 \pm 2.4)	3/3 (6.9 \pm 1.4)	1/3	3/3	1.21

^a Oseltamivir 10 or 25 mg/kg/day or sterile PBS (control animals) was given twice daily for 5 days, starting 24 h after inoculation with 10^2 EID₅₀ of A/Vietnam/1203/04 (H5N1) virus.

^b The mean day to death of ferrets was determined by the Kaplan-Meier method. *, $P < 0.05$, one-way ANOVA.

^c The maximal change in the mean value (%) \pm SD is shown in parentheses. *, $P < 0.05$, one-way ANOVA.

^d Respiratory signs were sneezing, wheezing, and nasal discharge.

^e Neurological signs were hind limb paresis, ataxia, torticollis, and tremors.

^f Determined after a 14-day observation (35). Control ferrets were observed for only 8 days because of mortality.

mg/kg/day of oseltamivir was applied. However, this drug regimen significantly inhibited virus replication in the lungs, liver, and spleen ($P < 0.05$). Treatment with 25 mg/kg/day of oseltamivir completely inhibited virus replication in the internal organs of one of the two animals. In the other, virus was detected only in the brain (in two of two samples).

Efficacy of delayed treatment in ferrets inoculated with A/Turkey/15/06 (H5N1) virus. To assess the efficacy of oseltamivir against a less pathogenic H5N1 influenza virus, we inoc-

ulated ferrets with 10^6 EID₅₀ of A/Turkey/15/06 virus and began treatment with 10 mg/kg/day of oseltamivir 24 h later. Inoculated control ferrets showed relatively mild signs of illness (Table 4). The ferrets that received treatment with oseltamivir stayed more active throughout the observation period (relative inactivity index, 0.15), regained weight faster than untreated animals, and had a mean peak temperature increase 0.7°C lower than that in the control group (data not shown).

Although virus titers were little affected by delayed treatment with 10 mg/kg/day of oseltamivir (Fig. 4A), the peak nasal inflammatory cell counts in nasal washes were significantly lower in treated than in control animals on days 5 and 7 p.i. ($P < 0.05$). Moreover, cell counts remained at the same level in the control animals on days 3, 5, and 7 p.i., whereas they had returned to nearly normal levels in the treatment group on day 5 p.i. (Fig. 4B). Comparison of the protein concentrations in the nasal washes confirmed that there was significantly less upper respiratory tract inflammation in the treatment group (Fig. 4C).

To determine the effect of delayed treatment on virus spread to the lower respiratory tract and internal organs, we collected organs on day 6 p.i. Virus was detected in the lungs (in all four lobes assayed separately) of two of two control ferrets (Table 4). Immunostaining revealed virus-positive cells in tissue sections from the brains of control ferrets only (Fig. 2C). Importantly, virus was not detected in the lungs or other internal organs of ferrets treated with 10 mg/kg/day of oseltamivir either by virus titration in embryonated chicken eggs (Table 4) or by specific staining of the brain sections (Fig. 2D).

Rechallenge with a lethal dose of H5N1 virus after completion of oseltamivir treatment. To determine whether oseltamivir treatment alters antibody production and thus immune protection against infection with a new virus, we rechallenged the surviving treated ferrets with a lethal dose of A/Vietnam/1203/04 virus. After the first challenge, ferrets showed low HI titers (1:20 to 1:40) against homologous antigen but higher HI titers (1:80 to 1:160) against heterologous A/HK/213/03 (H5N1) virus (Table 5). This observation was consistent with a previous report showing that A/Vietnam/1203/04 virus elicited low detectable HI antibody titers in ferrets (18). Importantly, however, serum antibody production was sufficient to provide complete protection against lethal H5N1 virus challenge in this study.

Ferrets initially challenged with A/Turkey/15/06 (H5N1) vi-

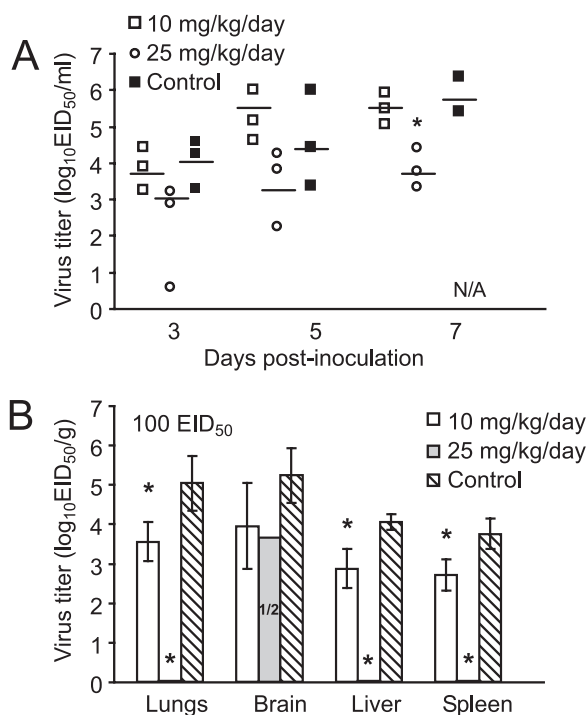


FIG. 3. Effect of delayed (24 h p.i.) oseltamivir treatment on virus titers in the nasal washes (A) and internal organs (B) of ferrets inoculated with 10^2 EID₅₀ of A/Vietnam/1203/04 (H5N1) virus. (A) Ferrets were treated with oseltamivir at 10 or 25 mg/kg/day or with sterile PBS (control animals) twice daily for 5 days. Nasal washes were collected on days 3, 5, and 7 p.i. Each point represents the result from a single ferret. Lines represent mean values of positive data (titers of >0.75 log₁₀ EID₅₀/ml, the limit of virus detection). N/A, not applicable (all ferrets in the group were dead). (B) Virus titers in the lungs, brains, livers, and spleens of ferrets at day 6 p.i. Values are means \pm SD for two ferrets. *, $P < 0.05$, one-way ANOVA.

TABLE 4. Effect of delayed (24 h p.i.) oseltamivir treatment on clinical signs of A/Turkey/15/06 (H5N1) virus infection in ferrets

Oseltamivir dose (mg/kg/day) ^a	No. of ferrets that survived/ total no.	No. of ferrets showing indicated clinical sign(s) of disease/total no.				Relative inactivity index ^f	Mean virus titer in indicated internal organ \pm SD ^b	
		Wt loss ^c	Increase in temp ^c	Respiratory ^d	Neurological ^e		Lungs	Brain
10	3/3	3/3 (17.9 \pm 2.8)	3/3 (6.0 \pm 0.9)	2/3	0/3	0.15	<*	<
0	3/3	3/3 (19.0 \pm 1.8)	3/3 (5.7 \pm 0.4)	1/3	0/3	0.46	2.9 \pm 0.7	<

^a Oseltamivir at 10 mg/kg/day or sterile PBS (control animals) was given twice daily for 5 days, beginning 24 h after inoculation.

^b Organs were collected on day 6 p.i. The values are virus titers determined in lungs or brains of animals and are expressed as log₁₀ EID₅₀/g. <, below the level of detection (<0.75 log₁₀ EID₅₀/g); *, $P < 0.05$, unpaired t test.

^c Maximal changes in the mean value (percentage) \pm SD are shown in parentheses.

^d Respiratory signs were sneezing, wheezing, and nasal discharge.

^e Neurological signs were hind limb paresis, ataxia, torticollis, and tremors.

^f Determined after a 14-day observation (35).

rus showed homologous HI titers of 1:160 to 1:320 (Table 5). Animals were completely protected from rechallenge with 10⁷ EID₅₀ of the virus; they showed no disease signs, and none shed virus on day 3 p.i.

Emergence of oseltamivir-resistant variants during treatment. To detect the emergence of oseltamivir-resistant mutants during treatment, we extracted viral RNA directly from the nasal washes and internal organs of ferrets and sequenced the NA and HA (HA1 subunit) genes. Direct sequence analysis was first used to identify the dominant virus population. Sequencing of the samples obtained on days 5 to 7 p.i. from ferrets treated prophylactically with 5 mg/kg/day of oseltamivir revealed only one amino acid substitution (I418M) in a virus isolated from the brain of a single animal inoculated with 10 EID₅₀ of A/Vietnam/1203/04 (H5N1) virus (Table 6). Residue 418 is located near the NA enzyme's active site and is not known to be associated with resistance to NA inhibitors. No changes were detected in the HA1 subunit. Plaque reduction assay in MDCK cells showed no change in the susceptibility of this sample to oseltamivir carboxylate.

Direct sequencing of the 18 samples obtained from ferrets that received delayed treatment with 10 or 25 mg/kg/day of oseltamivir revealed no mutations in the surface glycoproteins. Because direct sequence analysis might not detect minor populations of drug-resistant viruses (22), we analyzed virus clones obtained either from individual plaques in MDCK cells or from clones obtained after ligation of purified PCR products into a special TOPO vector. Sequence analysis of the 20 individual plaques obtained from the nasal wash sample of one ferret treated with 10 mg/kg/day of oseltamivir identified one clone with a V116A mutation in the NA gene and one clone with a V178I mutation in the HA1 region. Using the more rigorous technique, we analyzed three samples obtained from ferrets treated with oseltamivir: (i) a lung sample, (ii) a sample from the frontal region of the brain, and (iii) a nasal wash sample. We found NA amino acid changes in 1 of 10 clones (H274Y) sequenced from the lung sample of the ferret treated with 10 mg/kg/day of oseltamivir and in 1 of 10 clones (H274R, E277Q) sequenced from the brain sample of a ferret treated with 25 mg/kg/day of oseltamivir (Table 6). No other mutations were found in the nasal wash sample. Importantly, plaque reduction assays showed no change in the susceptibilities of those samples to NA inhibitor, although some resistant clones may have been present.

To detect the emergence of resistant variants of the less pathogenic A/Turkey/15/06 (H5N1) virus, we directly extracted RNA from 10 samples obtained on days 5 to 6 p.i. from nasal washes, lungs, and brains. We detected one mutation in the nasal wash sample of one ferret. This mutation, R193K in the HA1 region, did not result in a reduction of susceptibility to the NA inhibitor in vitro (Table 6).

DISCUSSION

Although stockpiling of antiviral drugs is an essential component of global influenza pandemic preparedness, there is too little information about their efficacy against potential H5N1 pandemic viruses. Here, we found that a low dosage of oseltamivir protects ferrets against lethal challenge with A/Vietnam/1203/04 (H5N1) influenza virus when treatment is started shortly after virus exposure. The virulence of this virus was a factor, requiring a higher oseltamivir dosage when treatment was delayed 24 h after the virus exposure. In ferrets inoculated with the less pathogenic A/Turkey/15/06 (H5N1) virus, oseltamivir inhibited inflammation in the upper respiratory tract and blocked the spread of virus to the internal organs.

H5N1 influenza viruses can cause systemic illness and neurological complications in multiple mammalian species (mice, ferrets, tigers, and leopards) (21, 28, 29). Our previous studies suggest that broad tissue tropism, high replicative efficiency, and neurovirulence are among the possible causes of the high lethality of H5N1/04 viruses in ferrets (10). These observations raised an important question: will antiviral drugs that are effective against contemporary seasonal human influenza viruses be effective against systemically replicating viruses? In the present study, we used representatives of two distinct clades of the H5 HA phylogenetic tree: A/Vietnam/1203/04 virus belonging to clade 1 and A/Turkey/15/06 virus belonging to clade 2, subclade 2 (46). These viruses differ dramatically in their pathogenicities to ferrets. Inoculation of ferrets with as little as 10 EID₅₀ of A/Vietnam/1203/04 (H5N1) caused lethal infection, with a systemic spread of the virus. Signs of upper respiratory disease (sneezing and nasal discharge) were not frequently observed when ferrets were infected with A/Vietnam/1203/04 (H5N1) influenza virus. Our studies do not permit us to definitively determine the cause of death of animals; however, we believe that virus quickly spreads to the lower respiratory tract and causes viral pneumonia. One of the causes of

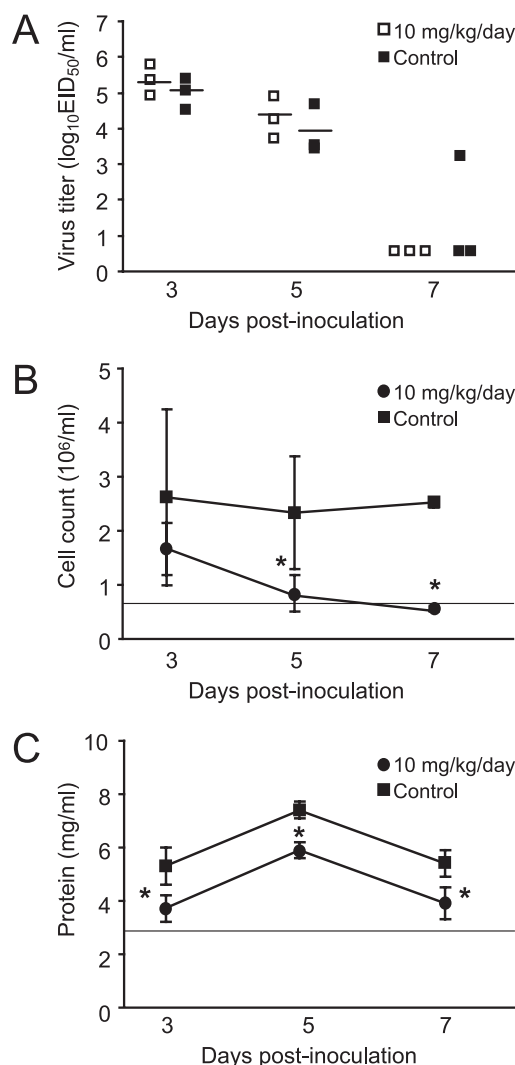


FIG. 4. Effect of delayed (24 h p.i.) oseltamivir treatment on virus titers (A) and inflammatory responses (B, C) in the upper respiratory tracts of ferrets inoculated with A/Turkey/15/06 (H5N1) virus. (A) Titers of nasal wash specimens collected on days 3, 5, and 7 p.i. from ferrets treated with 10 mg/kg/day oseltamivir or with sterile PBS (control animals) twice daily for 5 days. Each point represents the result from a single ferret. Lines represent mean values of positive data (titers of $>0.75 \log_{10} \text{EID}_{50}/\text{ml}$, the limit of virus detection). The total numbers of inflammatory cells (B) and the protein concentrations (C) in nasal washes collected at the indicated times were determined. Values are means \pm SD for three ferrets. *, $P < 0.05$, compared to the control value (unpaired two-tailed t test). The horizontal line (B, C) shows the mean values for uninfected animals.

death is probably viral pneumonia; however, virus replication in the ferrets' brains and neurological symptoms could also be a contributing factor in the deaths of animals. Conversely, 10^6 EID₅₀ of A/Turkey/15/06 (H5N1) virus caused only mild infection in ferrets, and 10 mg/kg/day of oseltamivir administered 24 h after virus exposure resulted in significantly less nasal inflammation and the absence of virus replication in the internal organs. Against A/Vietnam/1203/04 (H5N1), oseltamivir was not effective at a dosage of 10 mg/kg/day (equivalent to the approved human dose of 75 mg twice daily) (45) in preventing the deaths of animals when treatment initiated 24 h after virus inoculation, suggesting that the high virulence of this virus can affect requirements for higher drug dosages, as was observed previously with a mouse animal model (48). At 25 mg/kg/day, however, ferrets were protected from death. A/Vietnam/1203/04 (H5N1) virus is extremely pathogenic to mammalian hosts; in mice, it is more virulent than even the reconstructed 1918 Spanish influenza virus (29, 42).

Another important question is drug efficacy against neurovirulent H5N1 influenza viruses. Encephalitis and encephalopathy have been reported to occur at low frequencies in patients infected with contemporary H1N1 and H3N2 human influenza viruses, whereas severe human cases of H5N1 infection are associated with far higher rates of disseminated disease, viremia, and encephalitis (41, 43, 49). Infectious A/Vietnam/1203/04 (H5N1) virus has previously been isolated from the brains of infected ferrets (10, 29). Our immunostaining studies revealed massive viral invasion of the brain. We found that oseltamivir can affect virus titers in the brains of infected animals, depending on (i) the dose, (ii) the time of initiation of treatment, and (iii) the pathogenicity of the virus. Early initiation of treatment appears to be crucial for effective drug treatment. When we administered oseltamivir 4 h after inoculation with A/Vietnam/1203/04 (H5N1) virus, even half the approved human treatment dose protected animals against death. However, virus was detected in the brain of one of the two animals tested. The mechanism of oseltamivir action against neurovirulent viruses is not completely understood. Available information suggests that oseltamivir has a limited ability to cross the brain-blood barrier (39), and therefore penetration of oseltamivir into the brain and inhibition of virus replication in situ are also limited. However, we can speculate that penetration of the blood-brain barrier may be compromised in severely infected animals and therefore may differ from that in uninfected animals. There is an urgent need to

TABLE 5. Results of rechallenge of ferrets with a lethal dose of homologous H5N1 virus after completion of oseltamivir treatment

Virus	Oseltamivir regimen	Rechallenge virus dose (EID ₅₀ /ferret) ^a	Range of prechallenge HI titers ^b			No. of ferrets that survived/total no.	No. of ferrets with clinical signs of disease/total no. ^c
			A/Vietnam/1203/04	A/HK/213/03	A/Turkey/15/06		
A/Vietnam/1203/04	25 mg/kg/day, 24-h delay	10^2	20–40	80–160	NT	3/3	0/3
A/Turkey/15/06	10 mg/kg/day, 24-h delay	10^7	20–40	320–640	160–320	3/3	0/3

^a Three weeks after the initial H5N1 virus inoculation, surviving oseltamivir-treated ferrets were reinoculated with 10^2 EID₅₀ of A/Vietnam/1203/04 or 10^7 EID₅₀ of A/Turkey/15/06 virus.

^b HI titers against homologous and heterologous viruses 3 weeks after initial inoculation (expressed as reciprocal values, e.g., 40 versus 1:40). NT, not tested.

^c Clinical signs of disease were weight loss, temperature increase, respiratory signs, and inactivity index. Nasal washes were collected on day 3 p.i. All were below the level of detection ($<0.75 \log_{10} \text{EID}_{50}/\text{ml}$).

TABLE 6. Emergence of resistant viruses during oseltamivir treatment

Virus and virus dose (EID ₅₀ /ferret)	Oseltamivir regimen	Origin of sample	Amino acid change (no. of clones/total no. sequenced) in ^a :		Mean EC ₅₀ ± SD (nM) from plaque reduction assay ^b
			NA	HA (HA1)	
A/Vietnam/1203/04					
10 ²	0 mg/kg/day	Nasal wash			0.5 ± 0.1
10	4-h delay, 5 mg/kg/day	Brain	I418M	—	0.5 ± 0.1
10 ²	5 mg/kg/day	Brain	—	—	NT
		Liver	—	—	NT
		Spleen	—	—	NT
10 ²	24-h delay, 10 mg/kg/day	Nasal wash	V116A (1/20)	V178I (1/20)	0.5 ± 0.1
		Lungs	H274Y (1/10)	—	0.5 ± 0.1
10 ²	24-h delay, 25 mg/kg/day	Brain	H274R (1/10) E277Q (1/10)	NT ^c	0.5 ± 0.1
A/Turkey/15/06					
10 ⁶	0 mg/kg/day	Nasal wash			32.2 ± 0.1
10 ⁶	24-h delay, 10 mg/kg/day	Nasal wash	—	R193K	27.7 ± 0.4

^a Direct RNA extraction and sequence analysis did not detect amino acid changes. Therefore, in five samples, NA and HA (HA1) amino acid mutations were identified by TOPO TA cloning or sequence analysis of virus clones obtained from individual plaques grown in MDCK cells from nasal washes or internal organs of ferrets. Amino acid numbering is based on N2 NA and on H3 HA (32). —, no mutations detected.

^b Oseltamivir dose required to reduce plaque size by 50% in MDCK cells infected with samples obtained on days 5 to 7 of oseltamivir treatment. Analysis was performed directly with a virus-containing sample or after one passage in MDCK cells.

^c NT, not tested.

determine the mechanism and drug regimen that optimally blocks virus penetration of and replication in the brain.

The relevance of animal models to human infection is always open to question. The ferret model is advantageous for studies such as this one, in that it allows an evaluation not only of virological parameters but also of clinical signs of infection. Moreover, receptor distributions in the airway epithelium (25), immune responses (36), and histopathologic changes (50) are similar in ferrets and in humans. Influenza H5N1 viruses differ in their pathogenicities to ferrets (10, 29, 50), and this animal model allows us to study drug efficacy against lethal H5N1 infection as well as against symptomatic manifestations of less pathogenic virus. Considering that not all human cases of H5N1 virus infection are fatal, an evaluation of drugs against viruses of different pathogenicities may provide the most useful information.

In our rechallenge experiments, animals were completely protected from lethal challenge with homologous H5N1 virus. Delayed oseltamivir treatment decreased the virus load but did not completely protect from illness. Therefore, oseltamivir treatment did not interfere with the development and maintenance of immunity to homologous H5N1 virus. Additional studies are needed to determine whether this level of serum antibodies is sufficient to protect against antigenically dissimilar H5N1 viruses. The ferrets used in the experiments had serum antibodies against influenza virus of the H3 HA subtype before inoculation with H5N1 virus. It is possible that initial infection with H3N2 virus provided some cross-protection against heterologous subtypes, but there are no experimental data that support this possibility. Further, humans infected

with H5N1 influenza viruses have antibodies to contemporary influenza viruses.

Emergence of resistant variants during the course of antiviral therapy has not been addressed extensively with animal models (16, 20, 48). The mouse model is the preferred choice for antiviral studies, although it may not be optimal for identifying the emergence of resistant variants due to different receptor specificities in mice and humans (19). Ferret tracheal epithelial cells express primarily sialic acid α 2,6 galactose receptor structures and a lesser amount of sialic acid α 2,3-galactose receptors (25). Therefore, this model more closely represents the human airway epithelium and allows the study of NA and HA mutations that may emerge during oseltamivir treatment. Aside from the choice of model, the best method of assessing the emergence of resistant variants is unclear. Our direct sequencing of samples did not detect NA or HA amino acid changes that might confer resistance. A more sophisticated analysis was required to detect a mixture of clones, one of which carries NA or HA mutations. Mutations at position 274 of NA are of major concern, as the therapy-associated emergence of oseltamivir-resistant H5N1 clones that had an H274Y NA mutation was recently described (8, 24). We identified such a mutation in 1 of 10 clones sequenced; therefore, the clones that carried an NA mutation were a small proportion of the overall virus population analyzed, and we found no change in oseltamivir susceptibility. There is currently no reliable cell culture-based system for in vitro susceptibility testing of resistant variants. The significance of a minor population of drug-resistant clones is unclear and requires further study.

In conclusion, these studies demonstrated that the NA in-

hibitor oseltamivir was effective against infection by two different clades of H5N1 influenza viruses in ferrets. A more pronounced effect was observed when treatment started early after virus exposure and thus highlighted the importance of timing in the use of oseltamivir. Unfortunately, antiviral treatment, when used for H5N1 virus infection in humans, was initiated late in the course of disease and did not provide a significant impact on clinical signs or mortality. Moreover, late initiation of therapy may not allow effective reduction of the virus load and might allow the selection of drug-resistant mutants. Therefore, we must develop strategies which can provide the earliest possible administration of antiviral drugs at appropriate dosages to individuals infected or strongly suspected of being infected with H5N1 influenza virus and to those individuals at high risk of infection based upon significant and unprotected contact with infected humans, animals, or laboratory samples.

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